Volume 314, number 2, 179–182 FEBS 11859 December 1992
© 1992 Federation of European Biochemical Societies 00145793/92/$5.00

# A mechanism for NADPH inhibition of catalase compound II formation

A. Hillar and P. Nicholls

*Department of Biological Sciences, Brock University, St Catharines, Ontario, L2S 3A1, Canada*

Received 12 October 1992

Catalase-bound NADPH both prevents and reverses the accumulation of inactive bovine liver catalase peroxide compound II generated by 'endogenous' donors under conditions of steady $H_2O_2$ formation without reacting rapidly with either compound I or compound II. It thus differs both from classical 2-electron donors of the ethanol type, and from 1-electron donors of the ferrocyanide/phenol type. NADPH also inhibits compound II formation induced by the exogenous one-electron donor ferrocyanide. A catalase reaction scheme is proposed in which the initial formation of compound II from compound I involves production of a neighbouring radical species. NADPH blocks the final formation of stable compound II by reacting as a 2-electron donor to compound II and to this free radical. The proposed behaviour resembles that of labile free radicals formed in cytochrome *c* peroxidase and myoglobin. Such radical migration patterns within haem enzymes are increasingly common motifs.

NADPH; Catalase, Compound II, Peroxide; Radical, Ferrocyanide

## 1. INTRODUCTION

Eukaryotic catalase is still an enzyme of uncertain functional status [1,2] despite much work. It may be physiologically useful in removing slowly generated hydrogen peroxide from the cell. Its peroxisomal location suggests a role in dealing with peroxides produced by peroxisomal oxidases [3]. In catalysing peroxide decomposition the enzyme cycles between a ferric 'ground' state and compound I, an $Fe^{V}$ state probably comprising a ferryl iron and a porphyrin radical structure [4]. However, the intermediate $Fe^{VI}$ redox state, compound II, an essential intermediate in peroxidase activity, is an inhibited form in the catalase case [5,6]. The activity changes from the 'α' to the 'β' state if the duration of peroxide contact is long enough [7].

Recently, tightly bound $NADP^+$ and NADPH were discovered [8] in catalase preparations that had not only been studied functionally for years [1,2,5] without recognition of such extra prosthetic groups but the structure of which had been determined by X-ray diffraction. The latter was reinterpreted in the light of the new discovery [9,10]. Bound NADPH is involved in protecting the enzyme from transformation into compound II [11]. But this event is a single electron one, while the oxidation of NADPH to $NADP^+$ is a two-electron process.

*Correspondence address:* P. Nicholls, Department of Biological Sciences, Brock University, St. Catharines, Ontario, L2S 3A1, Canada. Fax. (1) (416) 682 9020.

*Abbreviations:* Compound I, catalase peroxide compound I; Compound II, catalase peroxide compound II; NADP, nicotinamide adenine dinucleotide phosphate.

It was therefore proposed that NADPH bound to catalase may undergo one-electron oxidations [11,12]. Such a one-electron oxidation route would be unique for this coenzyme, at least under physiological conditions. Typically, one-electron donors promote rather than inhibit compound II formation [2]; nitrite, an exception [13], is effective because it reacts both with compound I as a two-electron donor and with compound II as a one-electron donor. It is unlikely that NADPH will have the promiscuity shown by nitrite. We therefore reexamined the NADPH behaviour to reclassify its activity in terms of the original donor classification of Keilin and Nicholls [2].

This paper will try to show that this effort failed and that NADPH, as well as NADH, is a member of a new class of catalase hydrogen donors with a unique mode of action towards the eukaryotic (beef liver) enzyme. This mode of action is functionally significant, and a role for NADPH binding is thus established.

## 2. MATERIALS AND METHODS

Catalase was the beef liver enzyme (Sigma C-100). Some control experiments were carried out with *E. coli* HPII catalase, courtesy of Dr Peter Loewen of the University of Manitoba. NADPH and $NADP^+$ were also Sigma products. Glucose was from BDH Chemicals, Poole, Dorset, UK. Glucose oxidase (*Aspergillus* enzyme) was Type X from Sigma. Other reagents were of AnalaR or similar quality throughout.

Spectrophotometry was carried out with a Beckman DU-50 instrument with microbeam attachment linked to a Comptech MSDOS-AT computer with an EGA graphics board with Beckman's Datalender software, and the results plotted on a Roland plotter. Semi-micro (1 ml, 1 cm) quartz cuvettes with black sidewalls were usually employed. Both complete spectral scans and continuous monitoring at 435 nm in the Soret region as in a previous study [11], were employed. Cata-

lytic assays were performed with this instrument or with a Beckman DU-7HS spectrophotometer.

## 3. RESULTS

Fig. 1 shows the effect of preaddition of NADPH upon the generation of bovine liver catalase compound II by 'endogenous' donors under conditions of steady $H_2O_2$ formation. The latter was achieved by providing the system with an appropriate mixture of glucose and glucose oxidase as in other investigations [5,6]. The enzyme shows a lag before compound II begins to be formed. During this phase the NADPH is oxidized to $NADP^+$ as seen by measurements at 340 nm (not shown).

The duration of the lag phase is proportional to the NADPH concentration when the latter has risen above a certain minimal level (Fig. 2), and is of the same order of magnitude as the time taken to produce an equivalent amount of compound II in the absence of NADPH. That is, the oxidation of NADPH proceeds at the same rate as that of compound II formation in the absence of NADPH. The latter does not react rapidly with either compound I or compound II, although it induces the slow decomposition of the latter at the same rate as ethanol (Fig. 3). Fig. 3A shows that a much larger concentration of ethanol than of NADPH is required to block compound II formation. The latter, if removing compound I at the same rate as ethanol, would have to react with a bimolecular rate at least 1,000 times greater than that of ethanol. The protection by a small quantity of NADPH could therefore not last longer than a few seconds before the coenzyme was turned into its fully oxidized form.

NADPH thus blocks compound II formation without reacting rapidly with either compounds I or II. The rate of compound II formation in Fig. 1 is limited by the

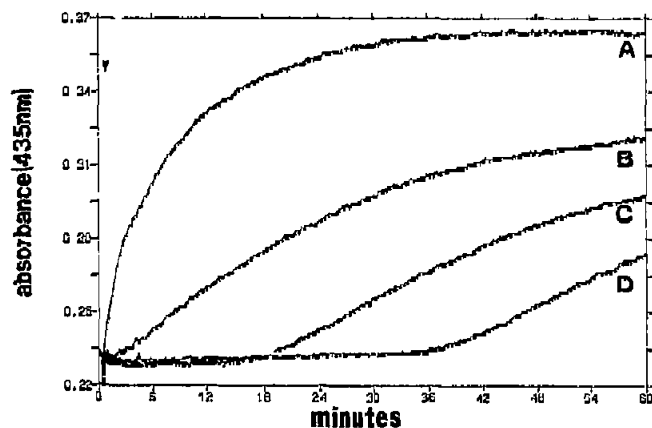


Fig. 1. Compound II formation by catalase in the presence of increasing amounts of NADPH Beef liver catalase (4.3 $\mu$M haematin) in aerobic 10 mM potassium phosphate buffer, pH 6.5 plus 2 nM glucose oxidase, 25°C, final vol. 1.0 ml. Effects of (A) 0, (B) 3.3, (C) 6.6 and (D) 10 5 $\mu$M NADPH indicated by the four traces. Glucose at 4 mM (arrowhead) initiated compound II formation.

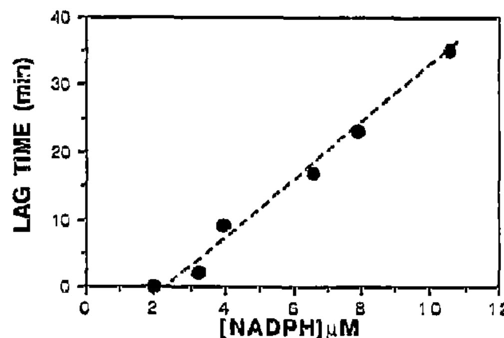


Fig. 2. Plot of the lag times in compound II formation against the initial concentration of NADPH present. Lag times from experiments analogous to those in Fig. 1 are plotted against the initial NADPH concentration. Beef liver catalase (≈4 3 $\mu$M haematin) was used in aerobic phosphate buffer, pH 6 5 with glucose oxidase and glucose at 25°C in a final volume of 1.0 ml as in the legend to Fig. 1.

activity of the 'endogenous one-electron donor' in the enzyme preparation [5,13]. The rate of compound II formation can be accelerated by addition of ferrocyanide, one of Keilin and Nicholls' Class I donors [2], as shown in Fig. 3B. NADPH also inhibits compound II formation induced by this exogenous one-electron donor.

## 4. DISCUSSION

NADPH effectively prevents accumulation of bovine liver catalase compound II generated by 'endogenous' donors or by the exogenous one-electron donor ferrocyanide under conditions of steady $H_2O_2$ formation, without reacting rapidly with either compound I or II. It thus differs both from 2-electron donors (ethanol-type), and from 1-electron donors (ferrocyanide/ascorbate type). That NADPH oxidation is itself no faster than compound II formation is obviously useful in preventing a waste of high energy reducing equivalents. But how is this physically possible? Kirkman et al. [11] suggested that NADPH engages in one-electron transfer events, although such behaviour would be almost unique in such a co-enzyme. Jouve and co-workers [12] have observed similar protective behaviour but do not proffer an explanatory model. In addition, De Sandro et al. [16] have recently also observed NADPH oxidation catalyzed rapidly by sequential one-electron transfers to compounds I and slowly via two-electron transfers to compounds II of horseradish peroxidase and 2,4-diacetyl-[$^2$H]heme-substituted horseradish peroxidase, upon single additions of hydrogen peroxide. Detailed mechanisms for these phenomena remain to be specified.

We propose the scheme in Fig. 4. This postulates a precursor to the usual compound II in which the oxidiz-

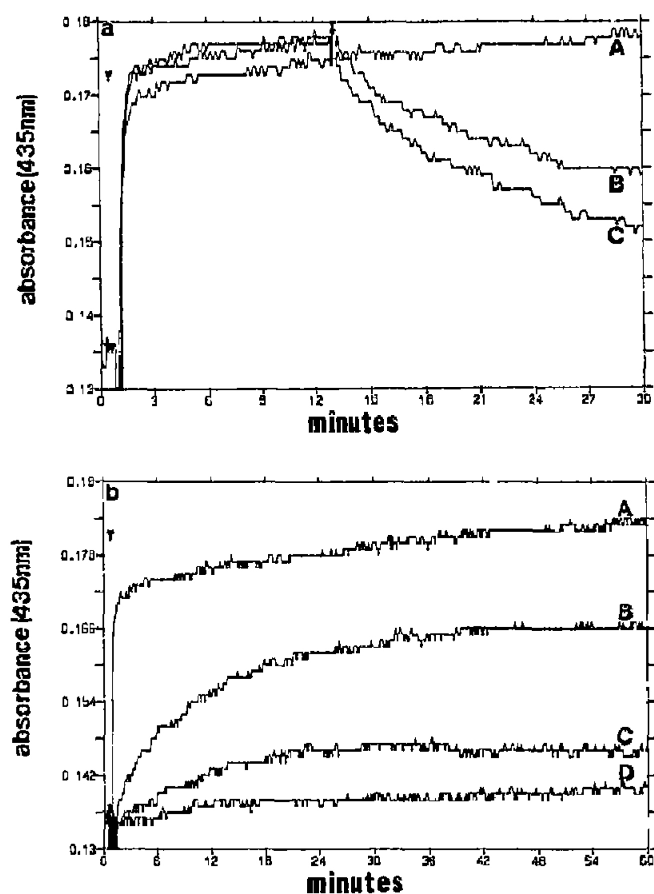


Fig. 3. Ethanol, ferrocyanide, and NADPH effects on compound II formation and decay. Beef liver catalase (2.0 $\mu$M haematin) in aerobic 10 mM potassium phosphate, pH 6.5, plus 2 nM glucose oxidase, at 25°C, final volume 1.0 ml. Glucose at 4 mM (single arrowheads) initiated compound II formation. (a) Ethanol- and NADPH-induced decompositions of compound II. Compound II formation in the presence of 11 $\mu$M $K_4Fe(CN)_6$. Additions of (B) 82 mM ethanol or (C) 17.5 $\mu$M NADPH as indicated (double arrowhead) with (A) serving as control. (b) Ferrocyanide-induced compound II formation ± NADPH. Compound II formation in the presence (A,C,D) or absence (B) of 11 $\mu$M $K_4Fe(CN)_6$ as indicated, with prior addition of (C) 82 mM ethanol or (D) 17.5 $\mu$M NADPH, with (A) serving as control

ing equivalent removed from the compound I porphyrin radical is still located close to the haem, rather like the corresponding radicals in cytochrome $c$ peroxidase [14] and myoglobin [15]. If nothing intervenes, the radical migrates elsewhere and eventually disappears by oxidizing some components of the system irreversibly. In the scheme, however, intervention occurs in the form of a reaction with bound NADPH. The latter is oxidized in a two-electron process distinct from the usual reaction with compound I. The two electrons are donated to the iron, reducing it to the ferric state, and to the radical, regenerating the original amino acid. This amino acid may be a tyrosine, as implicated in radical migrations in myoglobin. Several such tyrosines, in addition to the proximal haem ligand, are available on the distal side of the haem group, between it and the NADPH binding site.

The approximate catalase haem 'titration', followed by a stoichiometric relationship between NADPH concentration and lag time (Fig. 2) can then be explained as follows. The affinity of catalase for NADPH is quite high. Kirkman and Gaetani [8] have estimated a binding constant of the order of 10 nM. At equimolar NADPH concentrations and below, therefore, the bound coenzyme molecules only react with the ferryl iron–radical combination on their own subunit. Compound II formation on another subunit is unaffected provided that the rates of NADPH dissociation and migration to the new site are smaller than the rates of compound II formation and radical dissipation. No lag phase is seen, and compound II forms at a rate proportional to the concentration of unbound subunits. This is indeed the situation, as can be seen by inspection of results such as those in Fig. 1, as well as those obtained by previous workers [11,12].

Radical migration through a protein with time occurs with both myoglobin [15] and cytochrome $c$ peroxidase [14]. Between the NADP-binding site and the catalase haem group there is a $\beta$-sheet wall that contains a number of tyrosine residues. These may carry the oxidizing equivalents away from the haem as compound I decays to compound II and the latter to ferric enzyme [5]. The model (Fig. 4) assumes that the NADPH can reduce not only a labile tyrosine radical but also the remaining oxidizing equivalent on the iron. Whether this process is concerted or involves a radical intermediate as postulated by Kirkman et al. [11] remains uncertain. We prefer a concerted mechanism although the distance between NADPH and haem is considerable [9,10].

The NADPH effect applies to the exogenous donor ferrocyanide as well as endogenous donor (Fig. 3). As the active site must have lost one of the two oxidizing equivalents permanently after ferrocyanide reduction NADPH must react with the ferrocyanide-sensitive species before the latter has accepted the reducing equivalent. This implies that ferrocyanide reacts not with compound I directly but with the immediate decay product that leads to compound II. NADPH and ferrocyanide

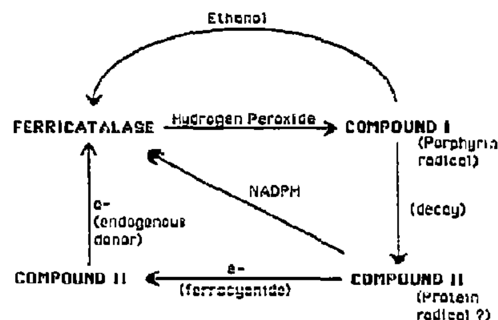


Fig. 4. Scheme for NADPH oxidation by catalase and protection of the enzyme from inactivation through compound II formation.

thus compete for this intermediate, the former catalysing a one-electron the latter a two-electron step. Ferrocyanide-induced compound II formation is considerably faster than that by endogenous donor (cf. Figs. 1 and 3B, kinetic traces, and ref. 13). Ferrocyanide may bind close to the haem and accelerate electron transfer while donating its electron slightly later; other anions have a similar effect upon compound II formation [5]. These phenomena are the subject of continuing study.

*Acknowledgements.* This work was supported by Canadian NSERC Grant A-0412 to P. Nicholls. We thank Dr. Peter Loewen for the *E. coli* HPII enzyme used in comparative fluorescence studies and for discussions of catalase activity.

## REFERENCES

[1] Fita, I. and Rossman, M G. (1985) J Mol. Biol 185, 21–37.
[2] Keilin, D. and Nicholls, P. (1958) Biochim Biophys Acta 29, 302–307.
[3] Thieringer, R., Shio, H., Han, Y., Cohen, G. and Lazarow, P.B (1991) Mol. Cell. Biol. 11, 510–522.
[4] Dolphin, D , Forman, A., Borg, D C., Fajer, J. and Felton, R.H. (1971) Proc. Natl. Acad. Sci. USA 68, 614–618.
[5] Nicholls, P. (1961) Biochem. J. 81, 365–374.
[6] Chance, B. (1950) Biochem. J. 46, 387–402.
[7] George, P. (1949) Biochem. J 44, 197–205.
[8] Kirkman, H.N. and Gaetani, G.F. (1984) Proc. Natl. Acad. Sci. USA 81, 4343–4347.
[9] Murthy, M.R.N., Reid, T.J., Sicignano, A., Tanaka, N. and Rossman, M.G. (1981) J. Mol. Biol. 152, 465–499.
[10] Fita, I. and Rossman, M.G. (1985) Proc. Natl. Acad. Sci. USA 82, 1604–1608.
[11] Kirkman, H.N., Galiano, S. and Gaetani, G.F. (1987) J. Biol. Chem. 262, 660–666.
[12] Jouve, H.M., Pelmont, J and Gaillard, J. (1986) Arch. Biochem. Biophys. 248, 71–79.
[13] Nicholls, P. (1964) Biochim. Biophys. Acta 81, 479–495.
[14] Fishel, L.A., Farnum, M.F , Mauro, J.M , Miller, M.A., Kraut, J , Liu, Y., Tan, X. and Scholes, C.P (1991) Biochemistry 30, 1986–1996
[15] Wilks, A. and Ortiz de Montellano, P.R. (1992) J. Biol. Chem. 267, 8827–8833.
[16] De Sandro, V., Dupuy, C , Kaniewski, J., Ohayon, R., Dème, D., Virion, A. and Pommier, J. (1991) Eur J. Biochem. 201, 507–513